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CRISPR/Cas9-mediated *ELANE* knockout enables neutrophilic maturation of primary hematopoietic stem and progenitor cells and induced pluripotent stem cells of severe congenital neutropenia patients

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ABSTRACT

Autosomal-dominant *ELANE* mutations are the most common cause of severe congenital neutropenia. Although the majority of congenital neutropenia patients respond to daily granulocyte colony stimulating factor, approximately 15 % do not respond to this cytokine at doses up to 50 µg/kg/day and approximately 15 % of patients will develop myelodysplasia or acute myeloid leukemia. “Maturation arrest,” the failure of the marrow myeloid progenitors to form mature neutrophils, is a consistent feature of *ELANE* associated congenital neutropenia. As mutant neutrophil elastase is the cause of this abnormality, we hypothesized that *ELANE* associated neutropenia could be treated and “maturation arrest” corrected by a CRISPR/Cas9-sgRNA ribonucleoprotein mediated *ELANE* knockout. To examine this hypothesis, we used induced pluripotent stem cells from two congenital neutropenia patients and primary hematopoietic stem and progenitor cells from four congenital neutropenia patients harboring *ELANE* mutations as well as HL60 cells expressing mutant *ELANE*. We observed that granulocytic differentiation of *ELANE* knockout induced pluripotent stem cells and primary hematopoietic stem and progenitor cells were comparable to healthy individuals. Phagocytic functions, ROS production, and chemotaxis of the *ELANE* KO (knockout) neutrophils were also normal. Knockdown of *ELANE* in the mutant *ELANE* expressing HL60 cells also allowed full maturation and formation of abundant neutrophils. These observations suggest that *ex vivo* CRISPR/Cas9 RNP based *ELANE* knockout of patients' primary hematopoietic stem and progenitor cells followed by autologous transplantation may be an alternative therapy for congenital neutropenia.

Introduction

Autosomal dominant *ELANE* mutations encoding neutrophil elastase (NE) are the most common cause of severe congenital neutropenia (CN), an inherited bone marrow failure syndrome.¹⁻³ Patients with CN suffer from severe life-threatening bacterial infections starting early after birth due to the absence or very low numbers of neutrophils in the peripheral blood (usually less than 500 cells per µL³).

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Hematopoietic stem and progenitor cells (HSPC) of CN patients fail to differentiate into mature neutrophils. This differentiation defect can be partially restored with daily or alternate-day subcutaneous injections of recombinant human granulocyte colony stimulating factor (rhG-CSF) in supra-physiological concentrations.⁴ Although rhG-CSF therapy improves the life expectancy and quality of life of CN patients, a subgroup does not respond to rhG-CSF. Additionally, about 15 % of CN patients developed myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) till now.³ There is a positive correlation between a rhG-CSF dose required to achieve acceptable neutrophil counts and a cumulative incidence to develop MDS or AML in CN patients.⁵ Therefore, CN patients, especially patients who either require high rhG-CSF dosages (above 50 µg/kg/day) and those who do not respond at all, need alternative therapeutic options. Hematopoietic stem cell transplantation (HSCT) would be a treatment of choice in CN patients, but it is associated with many adverse events, *e.g.* acute or chronic graft-versus-host-disease (GvHD), life-threatening infections, graft failure or graft rejection. Indeed, the overall survival of CN patients after HSCT is approximately 80 % only.

Recently established new technologies of CRISPR/Cas9-mediated gene editing in mammalian cells^{6,7} offer novel therapeutic options, especially for inherited monogenic disorders, including *ELANE* mutations associated CN. In this case, CRISPR/Cas9-mediated gene correction or knockout of the mutant gene in patient's HSPC *ex vivo* followed by autologous transplantation of the corrected HSPC might be a better treatment than high dose rhG-CSF or allogeneic stem cell transplantation.

ELANE mutations induce unfolded protein response (UPR) and endoplasmic reticulum (ER) stress in HSPC of CN patients that leads to increased apoptosis and defective granulocytic differentiation.⁸⁻¹¹ Therefore, inactivation of *ELANE* using CRISPR/Cas9-mediated knockout may abrogate UPR and ER stress caused by mutated *ELANE* with subsequent restoration of granulocytic differentiation. In support of this hypothesis, we recently identified a β-lactam-based inhibitor of human neutrophil elastase (NE), MK0339, which restored defective granulocytic differentiation of induced pluripotent stem cells (iPSC) and HL60 cells expressing mutated NE.¹² In addition, a recent report by Nayak *et al.* demonstrated the restoration of the *in vitro* granulopoiesis of *ELANE*-CN patient-derived iPSC upon treatment with Sivelestat, another NE-specific small-molecule inhibitor.^{12,13} Moreover, the fact that individuals showing mosaicism of inherited *ELANE* mutations have a higher proportion of *ELANE* mutated mature neutrophils hematopoietic cells in the bone marrow than in the blood^{14,15} supports the hypothesis that inactivation of *ELANE* mutations will improve neutrophil differentiation.

Another possibility to correct the disease phenotype is the direct correction of the specific gene mutation by the activation of homology-directed repair (HDR) of the mutated gene allele after cutting by CRISPR/Cas9 and co-transfection with a repair template. Most CN patients harbor inherited autosomal dominant missense or frameshift *ELANE* mutations that are distributed throughout all five exons and two introns.¹⁶ Therefore, CRISPR/Cas9-mediated correction of *ELANE* mutations would need to be patient/mutation specific. Since mutated *ELANE* may induce UPR and ER stress in edited cells, the introduction of new indels in the *ELANE* gene during the process of

CRISPR/Cas9 based editing may be not beneficial for the integrity of the hematopoietic stem cell (HSC) pool.

The first pre-clinical CRISPR/Cas9-based gene therapy study of common inherited blood disorders, sickle cell disease, and β-thalassemia, was reported.^{17,18} In these settings, the β-globin gene locus was inactivated by the introduction of deletions in autologous HSPC by CRISPR/Cas9-mediated gene editing. This was done to mimic the hereditary persistence of fetal hemoglobin mutations in HSC.^{17,18}

Here, we describe a CRISPR/Cas9 mediated *ELANE* KO by electroporation of HSPC and iPSC with *ELANE* specific CRISPR/Cas9-sgRNA ribonucleoprotein (RNP) complexes. *ELANE* KO induces granulocytic differentiation of HSPC and iPSC of CN patients harboring *ELANE* mutations without affecting their phagocytic functions. These results suggest that it may be possible to use CRISPR/Cas9 based *ELANE* KO in autologous HSCT as a therapy for *ELANE* associated neutropenia.

Methods

Patients

Three healthy donors and five severe congenital neutropenia patients harboring *ELANE* mutations (*ELANE*-CN) were used in the study. Bone marrow and peripheral blood samples from patients were collected in association with an annual follow-up recommended by the Severe Chronic Neutropenia International Registry. Study approval was obtained from the Ethical Review Board of the Medical Faculty, University of Tübingen. Informed written consent was obtained from all participants of this study.

Cell culture

Human CD34⁺ HSPC were isolated from bone marrow mononuclear cell fraction using Ficoll gradient centrifugation followed by magnetic bead separation using Human CD34 Progenitor Cell Isolation Kit, (Miltenyi Biotech, #130-046-703). CD34⁺ cells were cultured in a density of 2 × 10⁵ cells/mL in Stemline II Hematopoietic Stem Cell Expansion medium (Sigma Aldrich, #50192) supplemented with 10 % FBS, 1 % penicillin/streptomycin, 1 % L-Glutamine and a cytokine cocktail consisting of 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL TPO, 50 ng/ml SCF and 50 ng/mL FLT-3L (all cytokines were purchased from R&D Systems). Human induced pluripotent stem cells (iPSC) were cultured on Geltrex LDEV-free reduced growth factor basement membrane matrix (Thermo Fisher Scientific, #A1413201) coated plates in a density of 2 × 10⁵ cells/mL in StemFlex medium (Thermo Fisher Scientific, #A3349401) supplemented with 1 % penicillin/streptomycin. HL60 cells were maintained in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), 2 mM L-glutamine, and 1 % penicillin/streptomycin (Thermo Fisher Scientific) at 37°C and 5 % CO₂.

Design of the *ELANE*-specific guide RNA (gRNA)

Specific CRISPR-RNA (crRNA) for the knockout of the *ELANE* gene (cut site: chr19 [CTGCGCGGAGGC-CACTTCTG, +852,969 : -852,969], NM_001972.3 Exon 2, 161 bp; NP_001963.1 p.F54) was designed using the CCTop website.¹⁹

CRISPR/Cas9-gRNA RNP mediated *ELANE* KO in iPSC and HSPC

Electroporation was carried out using the Amaxa nucleofection system (P3 primary kit, #V4XP-3024) according to the manufacturer's instructions. 1×10^6 human iPSC or CD34⁺ HSPC were electroporated with assembled gRNA (8 μ g) and Cas9 (15 μ g) protein (Integrated DNA Technologies).

Isolation of single cell iPSC clones

8×10^3 human iPSC were plated on Geltrex-coated 10-cm dish in StemFlex medium (Thermo Fisher Scientific, #A3349401) and RevitaCell supplement (Thermo Fisher Scientific, #A2644501). The medium was changed every 24 hours without RevitaCell supplement. On day 7, single iPSC colonies were picked and transferred to the Geltrex-coated 96-well plates (one clone/well).

Colony Forming Unit (CFU) assay

CD34⁺ cells were resuspended in IMDM supplemented with 2 % FBS (Stemcell Technologies, #07700) and enriched Methocult (Stemcell Technologies, #H4435). The cell suspension was plated on 3.5 cm dishes (3×10^3 cells/dish) for 14 days.

In vitro phagocytosis assay

Cells were incubated with or without fluorescein-conjugated *Staphylococcus aureus* BioParticles (Invitrogen, #S2851) at a ratio of 100 particles per cell for two hours at 37°C, washed twice with PBS/ 2 % BSA, resuspended in 300 μ L FACS buffer and analyzed by flow cytometry.

Statistical analysis

Differences in mean values between groups were analyzed using two-sided, unpaired Student's *t*-tests using GraphPad Prism software.

Additional Material and Methods are available in the *Online Supplementary Material and Methods*.

Results

Inhibition of *ELANE* expression restored defective granulocytic differentiation of HL60 cell lines expressing endogenous *ELANE* mutations

We created CRISPR/Cas9 edited mutant *ELANE* knock-in HL60 human promyelocytic cell lines expressing either p.P139L or p.C151Y *ELANE* mutations. All-trans retinoic acid (ATRA) induced differentiation of wild-type and

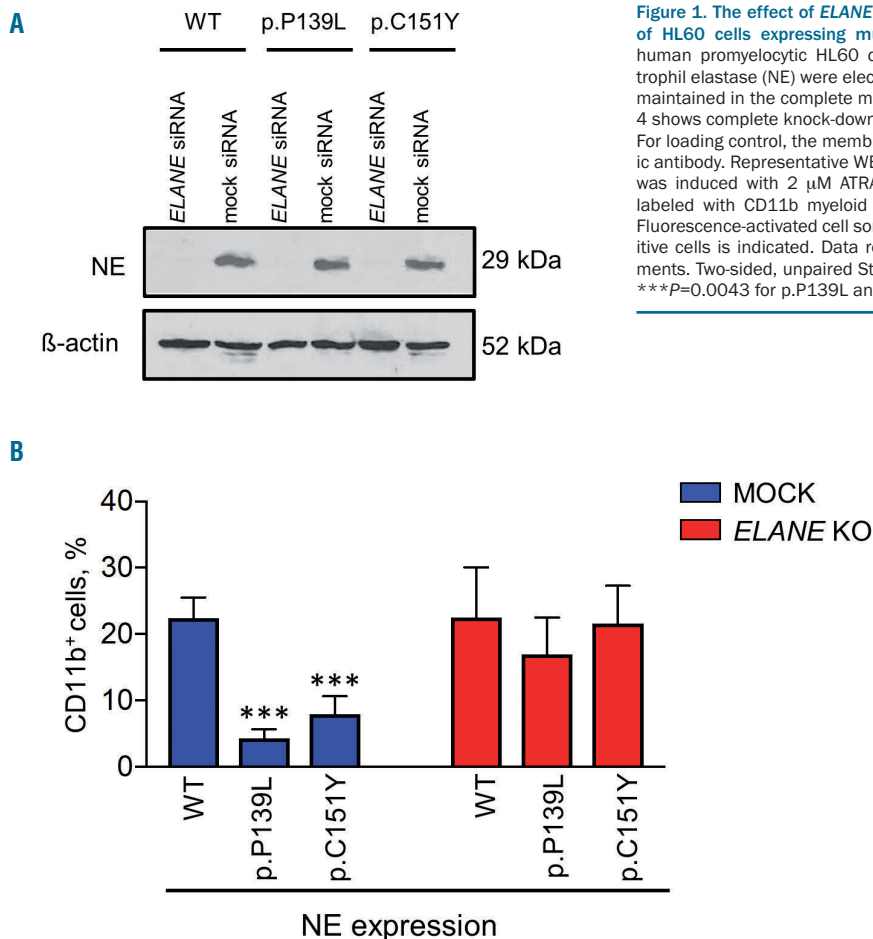


Figure 1. The effect of *ELANE* knock-down on the impaired myeloid differentiation of HL60 cells expressing mutant neutrophil elastase. (A) CRISPR/Cas9 edited human promyelocytic HL60 cells expressing p.P139L and p.C151Y mutant neutrophil elastase (NE) were electroporated with scrambled and anti-*ELANE* siRNA and maintained in the complete medium for five days. Western blot (WB) analysis at day 4 shows complete knock-down of NE detected with an anti-NE monoclonal antibody. For loading control, the membrane was stripped and re-probed with a β -actin-specific antibody. Representative WB membranes are depicted. (B) Myeloid differentiation was induced with 2 μ M ATRA (all-trans retinoic acid). After five days, cells were labeled with CD11b myeloid differentiation surface marker and examined using Fluorescence-activated cell sorting (FACS) analysis. The proportion of CD11b-PE positive cells is indicated. Data represent means \pm SD from four independent experiments. Two-sided, unpaired Student's *t*-test *P*-values are shown, ****P*<0.0001 and ****P*=0.0043 for p.P139L and p.C151Y respectively compared to wild-type (WT).

mutant HL60 clones revealed a typical impairment of granulocytic differentiation capacities in both mutant cell lines, as assessed by the significantly lower proportion of cells expressing CD11b granulocytic differentiation marker in p.P139L and p.C151Y mutant cell lines compared to the wild-type ($P < 0.0001$ and $P = 0.00043$, respectively) on day 5 of differentiation (Figure 1A-B and *Online Supplementary Figure S1A-C*). These findings are consistent with *ELANE* associated neutropenia patients phenotype.

As a proof-of-principle experiment, we have used RNA interference (RNAi) technology to knock down the expression of the *ELANE* gene in these cell lines. Thereby, we investigated the biological effects of inhibition of mutant NE on the granulocytic differentiation. Indeed, transfection of commercially available siRNA against the exon 4 of *ELANE*, completely knocked down the expression of NE in all cell lines. Production of CD11b positive cells was significantly restored in both mutant cell lines ($P = 0.00041$ for p.P139L and $P = 0.00048$ for p.C151Y), but

not in wild-type cells (Figure 1A-B and *Online Supplementary Figure S1A-C*).

Design and validation of sgRNA targeting *ELANE*

We further generated guide RNA (gRNA) specifically targeting exon 2 of *ELANE* by annealing CRISPR-RNA (crRNA) with trans-activating crRNA (tracrRNA). gRNA was incubated with recombinant Cas9 protein to generate CRISPR/Cas9-gRNA RNP complexes. The gRNA targeting exon 2 of *ELANE* (cut site: chr19[+852.969:-852.969], Figure 2A) was selected to introduce stop-codon mutations and to induce nonsense-mediated mRNA decay (NMD) of *ELANE* mRNA, which is caused by stop-codon or frameshift mutations at the beginning of *ELANE* mRNA. Based on our experimental analysis, the selected gRNA has high on-target activity with low off-target score (*data not shown*). To evaluate inhibition of NE expression by CRISPR/Cas9 RNP mediated targeting of exon 2 of *ELANE*, we generated an *ELANE* KO myeloid cell line

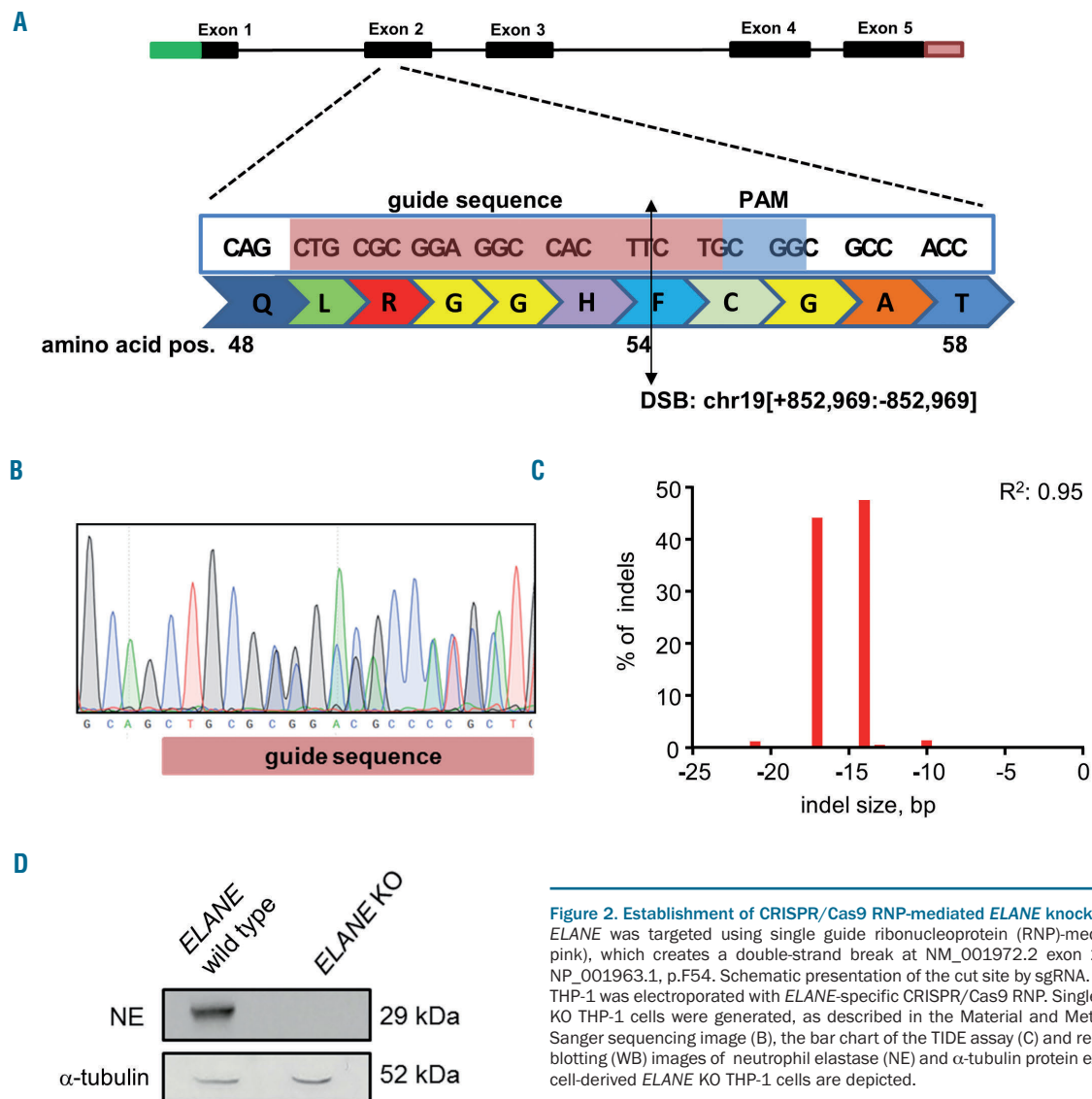


Figure 2. Establishment of CRISPR/Cas9 RNP-mediated *ELANE* knockout in THP-1 cells. (A) *ELANE* was targeted using single guide ribonucleoprotein (RNP)-mediated (highlighted in pink), which creates a double-strand break at NM_001972.2 exon 2, 161 bp after ATG; NP_001963.1, p.F54. Schematic presentation of the cut site by sgRNA. (B-D) Myeloid cell line THP-1 was electroporated with *ELANE*-specific CRISPR/Cas9 RNP. Single cell clones of *ELANE* KO THP-1 cells were generated, as described in the Material and Methods. Representative Sanger sequencing image (B), the bar chart of the TIDE assay (C) and representative Western blotting (WB) images of neutrophil elastase (NE) and α -tubulin protein expression (D) in single cell-derived *ELANE* KO THP-1 cells are depicted.

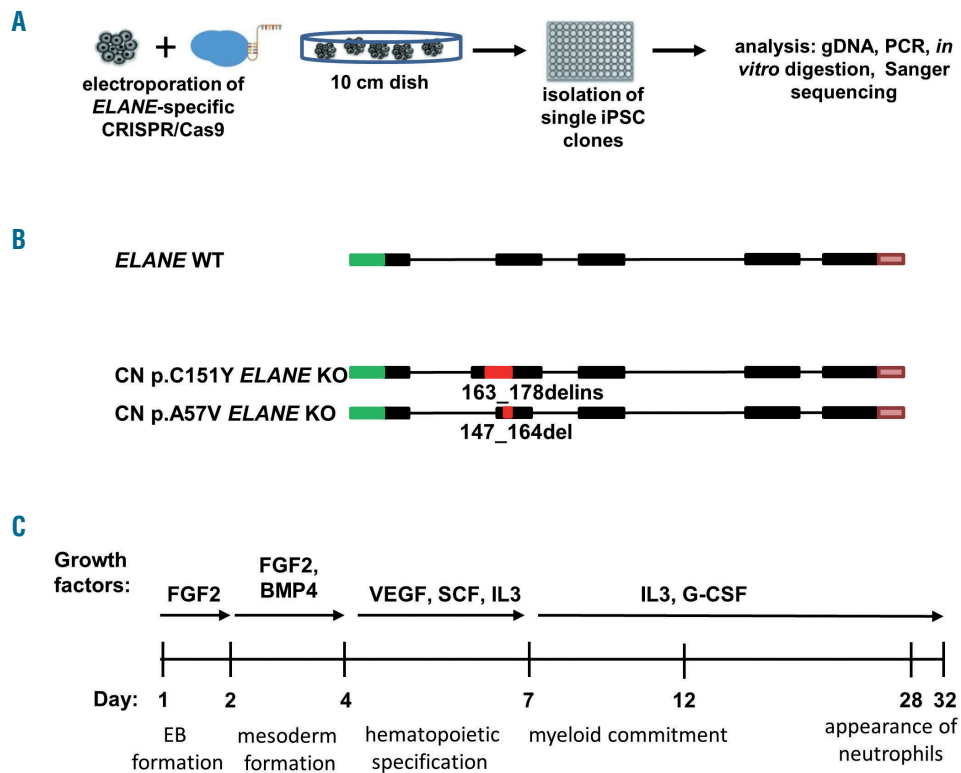


Figure 3. Generation of *ELANE* KO CN iPSC clones. (A) Scheme of the *ELANE*-specific CRISPR/Cas9-gRNA ribonucleoprotein electroporation of induced pluripotent stem cells (iPSC) and generation of *ELANE* KO iPSC clones. Generation of single *ELANE* knockout iPSC clones was made by seeding single iPSC, subsequent picking of each clone and transferring them into 96 well plates. Screening of each iPSC clone was done by Cas9 *in vitro* digestion and Sanger sequencing. (B) Scheme of CRISPR/Cas9 introduced modifications in the *ELANE* gene in iPSC clones of congenital neutropenia (CN) patients. Red inserts show positions of indels in *ELANE* mRNA (NM_001972.2) and numbers refer to bp position after ATG. (C) Scheme of the EB-based hematopoietic/neutrophilic differentiation of iPSC.

THP-1, which has high basal expression levels of *ELANE* and NE. The efficiency of *ELANE* knockout in the total population of edited THP-1 cells was 77 %, as assessed by Sanger sequencing and tracking of indels by decomposition (TIDE) analysis (*data not shown*). The pure *ELANE* KO THP-1 cell clone has compound heterozygosity of 14 and 17 bp deletions on each allele (Figure 2B-C). The NE expression was completely absent in the pure *ELANE* KO THP-1 cell clone, as determined by Western blotting (WB) using anti-NE antibody against the C-terminus of NE protein (Figure 2D, *Online Supplementary Figure S2A-B*). These data suggest that sgRNA targeting *ELANE* that we designed led to a complete loss of NE protein.

Restoration of the *in vitro* granulocytic differentiation in *ELANE*-CN iPSC clones after *ELANE* knockout

We generated iPSC from peripheral blood mononuclear cells (PB MNC) of two *ELANE*-CN patients, harboring *ELANE* mutations p.C151Y or p.A57V (CN p.C151Y iPSC and CN p.A57V iPSC, respectively). Additionally, iPSC of one healthy control (healthy ctrl iPSC) were evaluated. All three iPSC lines expressed elevated mRNA and protein levels of pluripotent stem cell-specific factors, displayed alkaline phosphatase activity and expression of pluripotent embryonic stem cell surface markers (*Online Supplementary Figure 3A-C*).

Next, we used electroporation of iPSC clones with *ELANE*-specific CRISPR/Cas9-sgRNA RNP to generate pure *ELANE* KO CN iPSC clones. For this, electroporated iPSC were seeded on a geltrex coated culture dish and single-cell derived iPSC clones were isolated transferred to geltrex coated 96 well-plates for the subsequent selection of *ELANE* knockout clones (Figure 3A). Confirmed *ELANE* knockout iPSC clones have followed *ELANE* modifica-

tions: 274 bp del/ins in CN p.C151Y *ELANE* KO iPSC, and 17 bp del in CN p.A57V *ELANE* KO iPSC (Figure 3B). The editing efficiency of healthy ctrl iPSC was 97 % (*Online Supplementary Figure S4A*), therefore, we used the total population of gene-edited healthy control (ctrl) iPSC for further analysis. We did not detect any off-target activity of the gRNA for the selected cDNA sites in all studied iPSC, as assessed using Sanger sequencing (*Online Supplementary Figure S4B* and *Tables S1, S2*).

Applying a slightly modified *in vitro* embryoid body (EB)-based iPSC differentiation method that allows generation of hematopoietic cells and mature myeloid cells for approximately 30 days,^{22,23} we found an increase in the percentage of CD15⁺CD16⁺CD45⁺ granulocytes in *ELANE* KO CN-iPSC cell culture, as compared to CN-iPSC. The generation of granulocytes from *ELANE* KO CN-iPSC was comparable to iPSC generated from a healthy donor (Figure 3C, 4A, and *Online Supplementary Figure S5A*). Generation of immature hematopoietic cells (CD34⁺KDR⁺, CD34⁺CD43⁺, CD45⁺CD235⁺CD41a⁺ and CD45⁺CD34⁺ cells) and CD45⁺CD33⁺ myeloid progenitor cells in *ELANE* KO CN- and CN-iPSC lines were similar or increased, in comparison to corresponding MOCK treated iPSC lines (*Online Supplementary Figure S6A*).

A CFU assay was performed with *ELANE*⁺ iPSC-derived CD34⁺ cells from CN patients and showed elevated levels of CFU-G but reduced CFU-M colony numbers, as compared to CD34⁺ cells derived from MOCK treated CN iPSC clones (Figure 4C). These data suggest that *ELANE* knockout restores granulocytic differentiation in CN.

We did not observe any significant defects in *in vitro* granulocytic differentiation of *ELANE* KO iPSC generated from a healthy donor, as compared to MOCK treated cells (Figure 4A-C, *Online Supplementary Figures S5* and *S6*).

ELANE knockout in HSPC of ELANE-CN patients restores diminished granulocytic differentiation

To further evaluate the clinical applicability of *ELANE* KO as a treatment option of *ELANE*-associated CN, we performed CRISPR/Cas9 RNP-mediated gene editing in primary bone marrow CD34⁺ HSPC of four *ELANE*-CN patients (Table 1) and three healthy donors and differentiated the cells towards neutrophils. *ELANE* knockout in CD34⁺ HSPC was performed by electroporation of human CD34⁺ HSPC with assembled *ELANE* specific sgRNA and Cas9 protein (Figure 5A). The editing efficiency varied between 27 % and 94 % (Figure 5B, *Online Supplementary Figure S7*). As expected, NE levels in neutrophils differentiated from the total population of edited cells were markedly reduced (Figure 5C-D, *Online Supplementary Figure S8A-B*). Moreover, *ELANE* KO leads to elevated granulocytic differentiation, as assessed by the percentage of CD15⁺CD11b⁺CD45⁺ cells (Figure 6A, *Online Supplementary Figure S9A-B*) and morphological examination of cytospin preparations of mature granulocytes generated on day 14 of the *in vitro* granulocytic differentiation using liquid culture (Figure 6B). At the same time, the ratio of *ELANE* KO cells increased from day 7 to day 14 of differentiation (Figure 5B). Simultaneously, the percentage of CD34⁺CD45⁺ cells was reduced in *ELANE* KO cells of CN patients, but not in healthy donor cells (*Online Supplementary Figure S10A*). In one patient (CN I120F), no difference in the percentage of CD15⁺CD11b⁺CD45⁺ cells between MOCK and *ELANE* KO samples was observed, but a clear improvement of granulocytic differentiation was detected in cytospin slides. This finding may be explained by relative mild neutropenia (*Online Supplementary Table 3*) and possible expression of CD15 in not fully mature myeloid cells in this patient.

Scanning and transmission electron microscopy revealed that *ELANE* KO cells of both healthy control and one CN patient showed no significant differences in morphology or intracellular structures, compared with MOCK cells of a healthy donor (Figure 6C-D).

Altogether, these data suggest that *ELANE* KO cells have a differentiation advantage over the HSPC carrying mutated *ELANE*.

Neutrophils generated from *ELANE* KO HSPC exhibited unaffected ROS production, phagocytosis and chemotaxis upon activation *in vitro*

We further evaluated *in vitro* activation of neutrophils generated from *ELANE* KO HSPCs in liquid culture for 14 days. We first performed an assessment of H₂O₂ levels (ROS) in fMLP-activated *ELANE* KO neutrophils generated from a healthy donor. We detected no differences between *ELANE* WT and *ELANE* KO neutrophils (Figure 7A).

Phagocytosis was evaluated by incubation of cells with fluorescein-conjugated *Staphylococcus aureus* BioParticles for two hours. Percentage of GFP⁺ granulocytes that engulfed bacteria were assessed by FACS using gating on granulocyte population in the dot plot of forward-scatter light (FSC) versus side-scatter light (SSC) channels. We did not detect any significant differences in phagocytosis of *ELANE* KO neutrophilic granulocytes, as compared to control MOCK cells (Figure 7B). As an independent evaluation of phagocytosis kinetics, we performed live cell imaging of neutrophils incubated with pHrodo Green *E. coli* Bioparticles Conjugate using IncuCyte ZOOM system and observed similar phagocytosis behavior of

MOCK and *ELANE* KO neutrophils generated from a healthy donor or one CN patient (Figure 7C).

Chemotactic activity of fMLP-treated neutrophils was also comparable between MOCK and *ELANE* KO groups (Figure 7D).

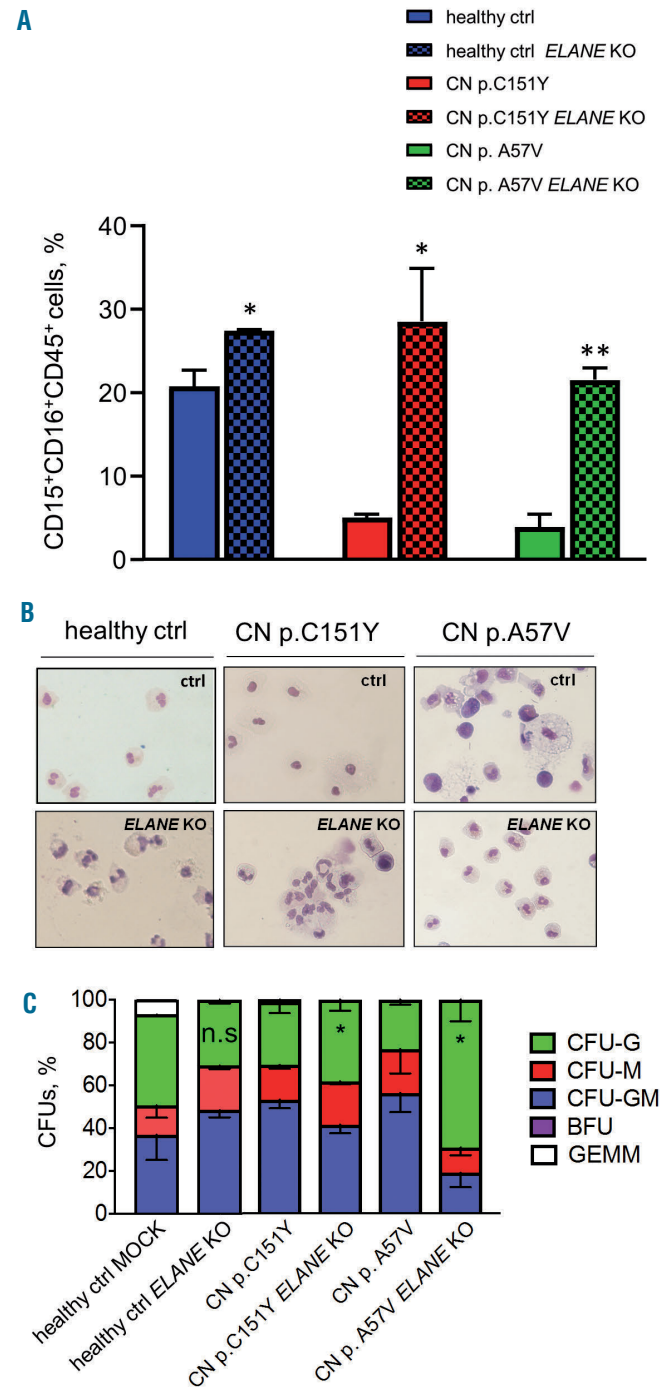


Figure 4. *ELANE* knockout restored granulocytic differentiation of *ELANE*-CN iPSC. (A) Flow cytometry analysis of suspension cells harvested from embryoid body (EB)-based granulocytic cell culture of respective iPSC clones on day 28 or 32 of differentiation. Data represent means \pm standard deviation (SD) from two independent experiments. * $P < 0.05$, ** $P < 0.01$. (B) Wright-Giemsa staining of cytospin preparations of suspension myeloid cells harvested from iPSC culture at day 28 or 32 of differentiation. Representative images are depicted. (C) Colony-forming unit (CFU) assay of CD34⁺ cells harvested from EB-based iPSC culture on day 14 of differentiation. Data represent means \pm SD from two independent experiments. * $P < 0.05$.

Unaffected phagocytic activity of *ELANE* KO PMN in zebrafish embryos

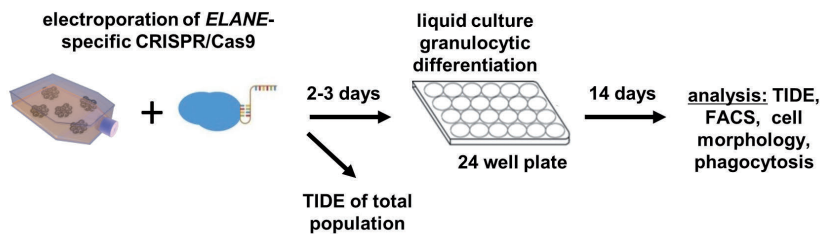
To evaluate the phagocytic activity of *ELANE* KO PMN *in vivo*, we transplanted fluorescently labeled polymorphonuclear leukocytes (PMN) generated from *ELANE* KO HD into zebrafish embryos (Figure 8A). PMN were injected into the duct of Cuvier, a wide circulation channel on the yolk sac connecting the heart to the trunk vasculature. Subsequently, Alexa-594-conjugated *Staphylococcus aureus* BioParticles were injected locally in the tail fin close to the caudal vein. Live imaging showed that human neutrophils migrated into the caudal hematopoietic tissue (CHT), which is equivalent to the fetal liver in mammals and provides a human-compatible environment.^{24,25} Confocal imaging of this region revealed that most of the *ELANE* KO neutrophils were found inside the perivascular pocket (Figure 8B) and many of them have engulfed bacteria (white arrows in Figure 8B and 8C, *Online Supplementary Movie S1*). Time-lapse *in vivo* imaging of the xenotrans-

planted embryos also revealed that human *ELANE* KO PMN have the capability to form surface protrusion within the perivascular region (Figure 8D, *Online Supplementary Movie S2*). We could not detect a difference between transplanted human *ELANE* KO and control MOCK PMN in zebrafish embryos (*data not shown*). These observations indicate that human *ELANE* KO PMN are able to migrate and phagocyte *in vivo*.

ELANE KO restores deregulated expression of UPR gene BiP and anti-apoptotic factor Bcl-x1 in *ELANE* KO iPSC derived cells of CN patients

We further evaluated the effects of *ELANE* KO on the expression of UPR gene BiP and anti-apoptotic factor Bcl-x1 (*Online Supplementary Figure S11*). We analyzed pure *ELANE* KO HSPC (for Bcl-x1) or neutrophils (for BiP) generated from iPSC of two CN patients. We found that *ELANE* KO HSPC express elevated mRNA levels of Bcl-x1 and BiP expression was markedly reduced in *ELANE* KO

A



B

| Sample, <i>ELANE</i> mutation position | Exon | Indel Efficiency, % | |
|--|--------|---------------------|--------|
| | | day 7 | day 14 |
| healthy ctrl 1 | - | 71 | 69 |
| healthy ctrl 2 | - | 57 | 46 |
| healthy ctrl 3 | - | 86 | 94 |
| CN p.A57V | Exon 2 | 52 | 63 |
| CN p.I120F | Exon 3 | 81 | 90 |
| CN p.S126L | Exon 4 | 45 | 57 |
| CN p.C223AfsX17 | Exon 5 | 27 | 37 |

C



D

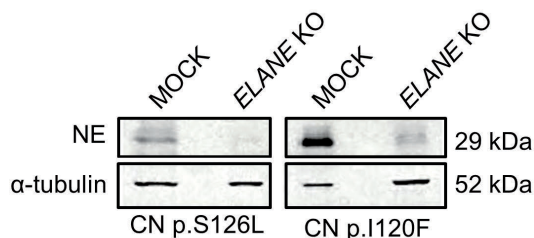


Figure 5. Efficient CRISPR/Cas9 RNP-based *ELANE* knockout in HSPC. (A) Scheme of the generation of *ELANE* KO HSPC using electroporation with *ELANE*-specific CRISPR/Cas9-gRNA ribonucleoprotein (RNP). (B) TIDE results of edited CD34⁺ HSPC at day 7 and 14 of liquid culture differentiation. (C and D) Hematopoietic stem and progenitor cells (HSPC) of healthy controls (C), or two CN patients (D) were electroporated with *ELANE*-specific CRISPR/Cas9 RNP, on day 14 of culture, cells were lysed in Laemmli buffer and Western blotting (WB) analysis using anti-neutrophil elastase (NE) antibody against C-terminus of NE was performed, staining with α -tubulin antibody was used as loading control. Representative WB images of cells from two independent experiments are depicted.

PMN, compared to cells carrying mutated *ELANE*. As expected, *ELANE* mRNA levels were severely diminished in *ELANE* KO cells (Online Supplementary Figure S11).

Discussion

The majority of patients suffering from congenital neutropenia respond well to daily treatment with rhG-CSF leading to a normal quality of life. However, in the last 25 years, we learned that CN is a preleukemic syndrome and that approximately 15 % of patients do not respond to

even ultra-high dosages (>50 µg/kg/d) of rhG-CSF. Therefore, we are searching for other treatment modalities for CN patients that may prevent leukemic transformation and may be useful for those who are requiring high dosages of rhG-CSF or not responding at all to rhG-CSF. For these patients, the only available treatment is stem cell transplantation with the risk of transplant-associated adverse events such as acute or chronic GvHD.

In the present study, we described for the first time the establishment of an *in vitro* cellular model of CRISPR/Cas9 mediated gene therapy of CN associated with autosomal dominant *ELANE* mutations, the most frequent cause of

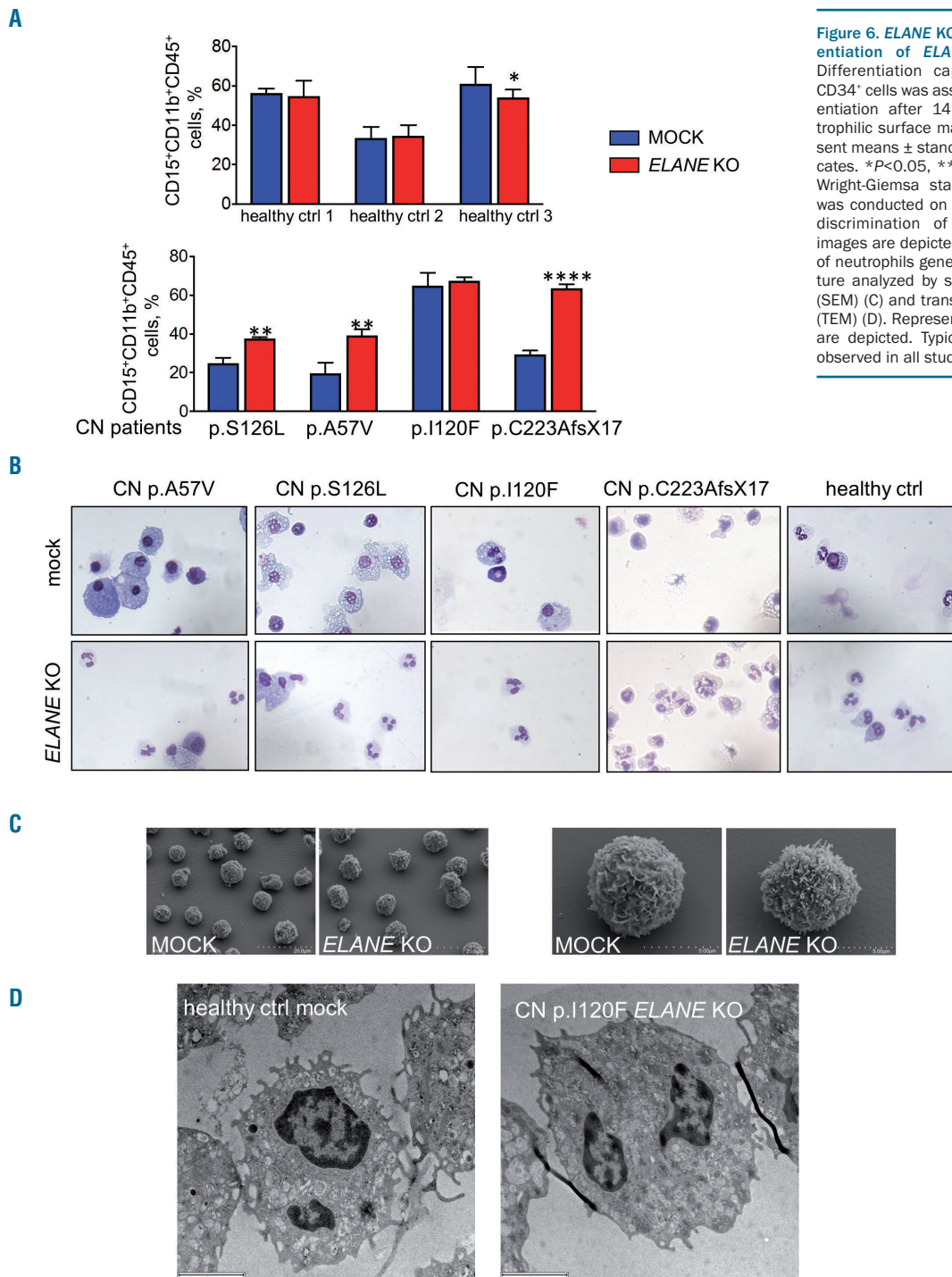


Figure 6. *ELANE* KO restored granulocytic differentiation of *ELANE*-CN primary HSPC. (A) Differentiation capacity of *ELANE* knockout CD34⁺ cells was assessed by liquid culture differentiation after 14 days by investigating neutrophilic surface marker expression. Data represent means ± standard deviation (SD) from triplicates. **P*<0.05, ***P*<0.01, *****P*<0.0001. (B) Wright-Giemsa staining of differentiated cells was conducted on day 14 allowing morphologic discrimination of the cells. Representative images are depicted. (C-D) Electron micrographs of neutrophils generated on day 14 of liquid culture analyzed by scanning electron microscopy (SEM) (C) and transmission electron microscopy (TEM) (D). Representative SEM and TEM images are depicted. Typical neutrophil morphology is observed in all studied samples.

CN. We tested *ex vivo* CRISPR/Cas9 RNP-based *ELANE* knockout in HSPC of CN patients that may be used for autologous transplantation as a therapeutic approach for *ELANE*-CN patients. Virus- and DNA-free application of CRISPR/Cas9 RNP markedly increases gene editing efficiency and simultaneously decreases the probability and frequency of off-target effects, because CRISPR/Cas9 RNP activity is preserved in cells for only approximately 48 hours. We recently reported the establishment of the fluorescent labeling of CRISPR/Cas9 RNP complexes for gene editing of primary hematopoietic stem cells and subsequent sorting of gene-modified cells for further applications.²⁶ Implementation of this method will improve the efficiency of gene knockout or gene correction in HSPC, including *ELANE* knockout or correction of *ELANE* mutations.

In case of *ELANE* KO, different combinations of the *ELANE* gene editing are expected: we may generate unedited, monoallelic edited (of mutated or WT allele), or bi-allelic edited HSPC. Since we did not use any selection marker for edited HSPC, we were not able to estimate the proportion of HSPC with inactivation of the mutated *ELANE* allele. The fact that the proportion of *ELANE* KO cells was elevated upon granulocytic differentiation strongly argues for the differentiation advantage of the edited cells lacking *ELANE* (including loss of the mutated allele).

There are several potential unforeseen consequences of the *ELANE* gene knockout strategy. For example, Tidwell *et al.* reported the presence of two in-frame ATG codons in exon 2 and exon 4 of *ELANE*.²⁷ They showed that the internal translation of NE can be initiated when the canon-

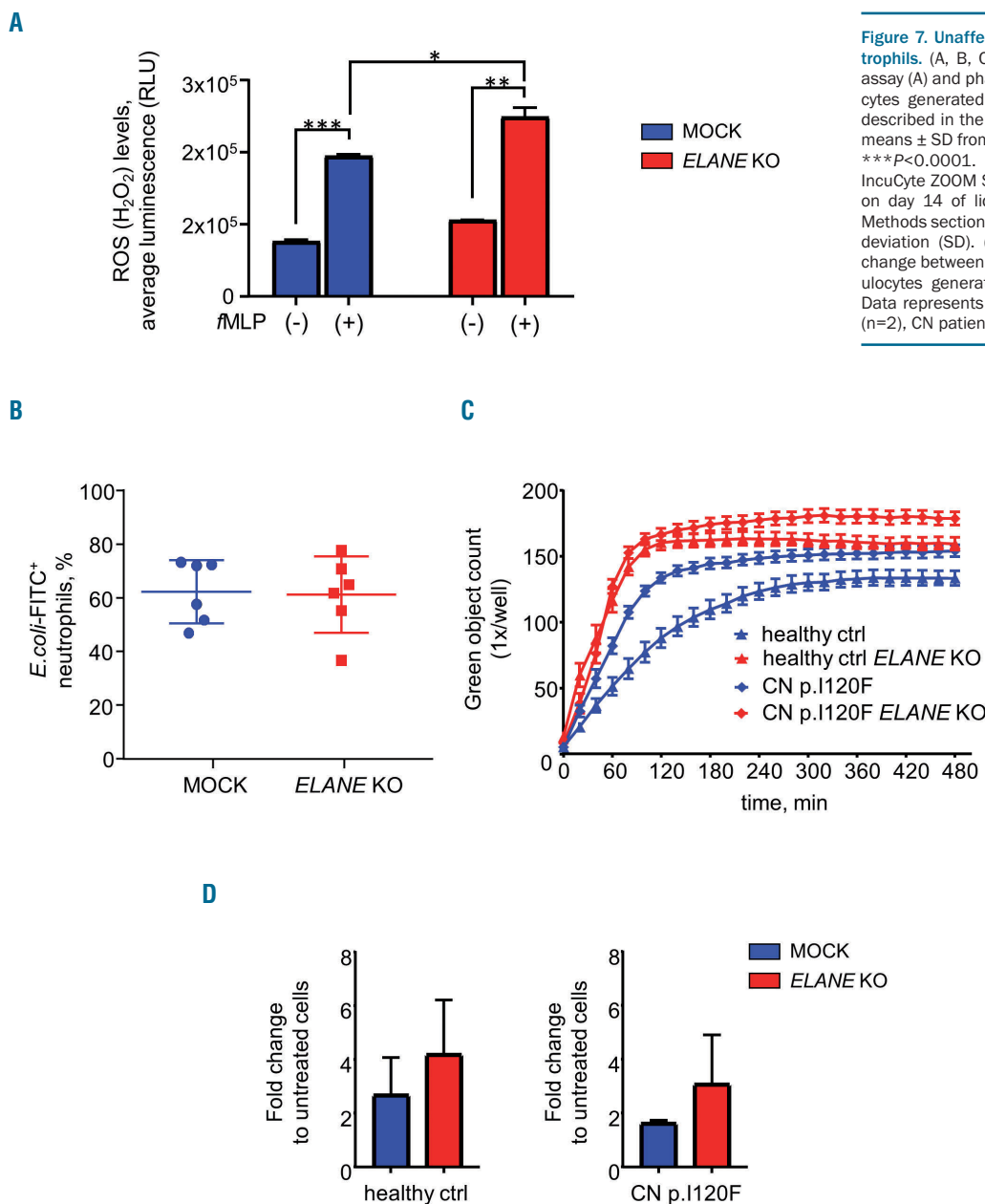


Figure 7. Unaffected functions of *ELANE* KO neutrophils. (A, B, C) Reactive oxygen species (ROS) assay (A) and phagocytosis assay (B, C) of granulocytes generated on day 14 of liquid culture, as described in the *Methods* section. Data represent means ± SD from duplicates. **P*<0.05, ***P*<0.01, ****P*<0.0001. (C) Phagocytosis Kinetic using InCuCyte ZOOM System of granulocytes generated on day 14 of liquid culture as described in the *Methods* section. Data represent mean ± standard deviation (SD). (D) Chemotaxis depicted as fold change between fMLP-treated and untreated granulocytes generated on day 14 of liquid culture. Data represents mean ± SD, healthy control (ctrl) (n=2), CN patient (n=1).

ical translational start site and/or internal start sites in exon 2 are disrupted and that expression of internally-initiated *ELANE* is pathogenic. We found a marked reduction of *ELANE* mRNA, most probably due to the induction of nonsense-mediated mRNA decay (NMD) of *ELANE* mRNA after exposure of cells to *ELANE*-specific CRISPR/Cas9 sgRNA RNP. We also did not detect any additional NE protein bands on WB analysis of edited cells using antibody recognizing C-terminus of NE. Based on these observations, we concluded that our sgRNA is inducing loss of NE protein without activation of the pathogenic *ELANE* forms from the internal ATG.

We did not detect off-target activity in edited cells, but recent results from Alan Bradley have suggested that the introduction of CRISPR/Cas9 editing can cause multiple genomic changes far beyond the actual target.²⁸ Therefore,

for clinical applications, evaluation of the off-target activity of CRISPR/Cas9 on whole genome level using next-generation sequencing should be performed. In addition, it would be important to evaluate that the editing of *ELANE* occurred in the repopulating hematopoietic stem cell population and that these cells maintained their ability to engraft immunodeficient mice *in vivo*. Since most probably HSC are not expressing NE, we will not expect any damaging effects of the *ELANE* KO on the functions and integrity of HSC.

We demonstrated here, that CRISPR/Cas9 mediated *ELANE* KO in HSPC and iPSC of CN patients induces granulocytic differentiation and *in vitro* generated *ELANE* KO neutrophils have no defects in the phagocytic activity, ROS production, and chemotaxis. NE is a proteolytic enzyme of the neutrophil serine protease (NSP) family,

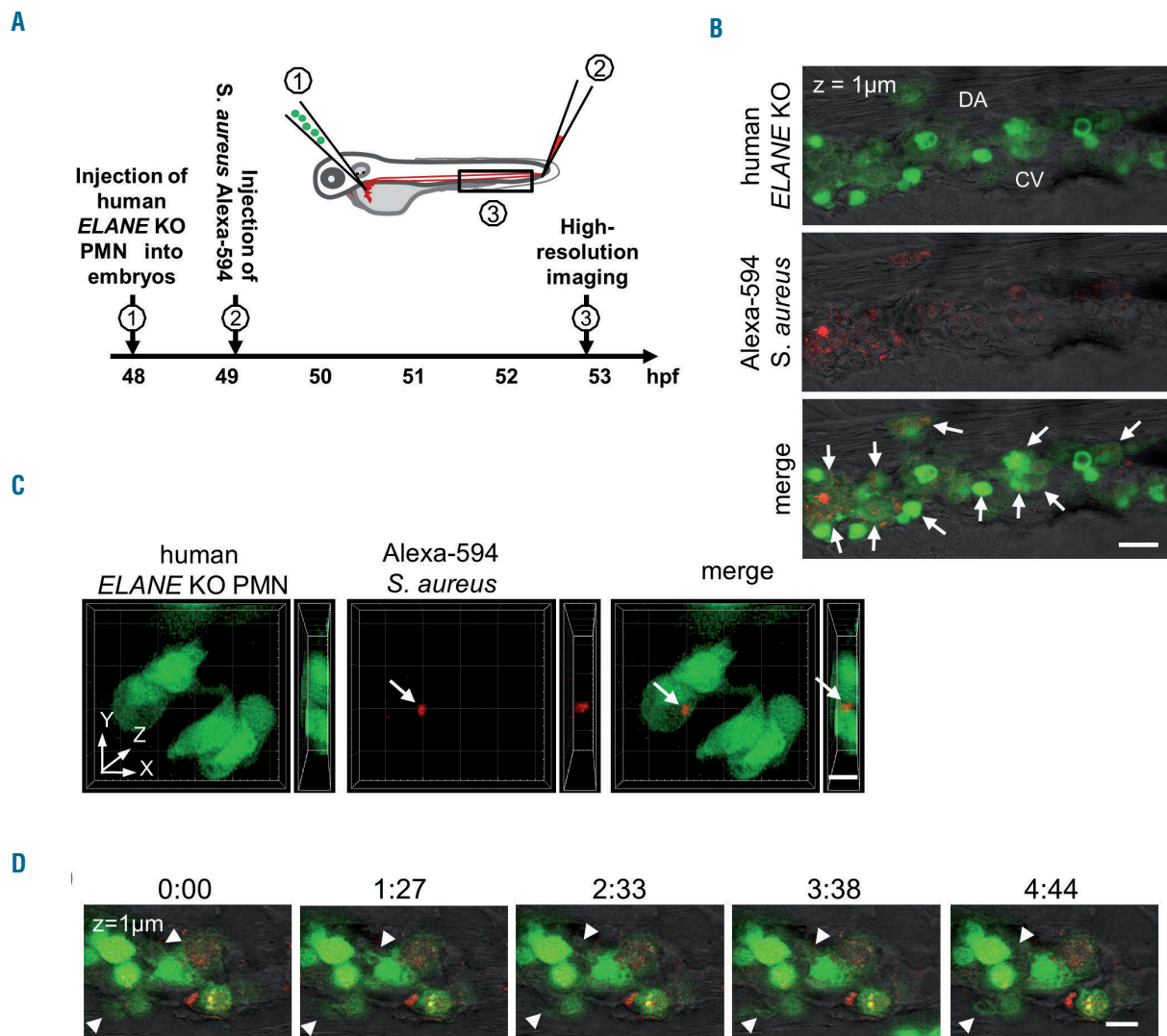


Figure 8. Human *ELANE* KO PMN are capable to migrate and to phagocytose *S. aureus* BioParticles in zebrafish embryos *in vivo*. (A) The scheme of *in vivo* phagocytosis assay in zebrafish embryos xenotransplanted with human fluorescently labeled polymorphonuclear leukocytes (PMN). (B) A representative confocal image highlighting the presence of transplanted human *ELANE* KO PMN in the caudal hematopoietic site of a zebrafish embryo at 53 hpf. White arrows indicate *S. aureus* BioParticles phagocytosed by human *ELANE* KO PMN. CV: caudal vein; DA: dorsal aorta. Scale bar: 20 μm. (C) Three-dimensional rendering of a z-stacks of 12 μm illustrating human *ELANE* KO PMN, one of them has engulfed *S. aureus* BioParticles (white arrow). Scale bar: 10 μm. (D) Still photographs from a time-lapse recording illustrating the phagocytic activity of transplanted human *ELANE* KO PMN in the zebrafish embryo. Arrowheads indicate the formation of neutrophil protrusions. Numbers indicate time in minutes. Scale bar: 10 μm.

including also cathepsin G (CG), proteinase 3 (PR3) and azurocidin (AZU1). NSP are stored in cytoplasmic granules, can be secreted into the extra- and peri-cellular space upon cellular activation and considered to be crucially involved in bacterial defense. NE and PR3 are very similar in their substrate specificity supporting a potentially redundant function for these two enzymes. *Elane*^{-/-} mice have normal neutrophil counts, but there are conflicting results regarding the effect of NE-deficiency on neutrophil extravasation to sites of inflammation, phagocytosis, and neutrophil extracellular traps in mice. NE may or may not be essential for these processes.²⁹⁻³³ Papillon-Lefevre Syndrome (PLS) is the only human disorder known to cause NE deficiency. This rare autosomal recessive disease is due to loss-of-function mutations in the DPPI gene locus with the loss of the lysosomal cysteine protease cathepsin C/dipeptidyl peptidase I (DPPI). The activation of NSP, including NE, depends on the N-terminal processing activity of DPPI. Therefore, PLS patients exhibit a severe reduction in the activity and stability of all three NSP including NE. Intriguingly, patients with PLS have no defects in their ability to kill bacteria e.g. *Staphylococcus aureus* or *E.coli*, suggesting that redundancies in the neutrophil's bactericidal mechanisms negate the necessity for serine proteases for killing common bacteria.³⁴ Moreover, since the other serine proteases including CG, PR3 and neutrophil serine protease 4 remain intact, we do not expect for the resultant cells to develop any neutrophil-specific functional anti-bacterial or immunodeficiency phenotype in *ELANE* KO cells. Based on these observations, at this juncture, we believe that CRISPR/Cas9 based knockout of *ELANE* in HSPC of CN patients may restore defective granulopoiesis in CN patients without seriously impairing neutrophil functions. Further studies, including gene expression analysis to understand which pathways are affected by *ELANE* mutations and verifying that these pathways are indeed restored by *ELANE* KO, are essential to justify the therapeutic applications of *ELANE* KO technology in the future. It will also contribute to the understanding of the pathophysiology of the CN caused by *ELANE* mutations. Our first attempts to investigate intracellular signaling pathways affected by mutated *ELANE* revealed the restoration of mRNA expression of anti-apoptotic Bcl-xl factor that is downregulated in CN myeloid progenitor cells.³⁵ Moreover, we found downregulation of mRNA levels of the key UPR player BiP, normally upregulated in HSPC of CN patients harboring *ELANE* mutations.⁸⁻¹⁰

CRISPR/Cas9 technology also allowed correction of the specific gene mutations. We selected the *ELANE* knockout approach since *ELANE* mutations are heterozygous gain-of-function gene defects that are distributed throughout all five exons and two introns of *ELANE* and specific *ELANE* mutations correction would require specific settings for each patient based on the mutation position. In addition, the requirement of the introduction of the donor repair template DNA in a gene correction approach

requires the activation of HDR making it difficult to achieve efficient correction in primary HSPC. We and other investigators have reported that *ELANE* mutations induce UPR and ER stress.⁸⁻¹¹ We also described deregulated signaling pathways in HSPC of CN patients downstream of *ELANE* mutations, such as diminished expression of transcription factors LEF-1, and C/EBP α ,³⁶⁻³⁹ abrogated expression and phosphorylation of the adaptor protein HCLS1,⁴⁰ elevated apoptosis³⁵ and hyperactivated NAMPT/sirtuins.⁴¹ These intracellular defects may lead to the elevated fragility of HSPC during *ex vivo* gene manipulations and may affect gene correction efficiency. Moreover, gene editing strategy directed to the correction of *ELANE* mutations may lead to the creation of novel missense or frameshift mutations that may result in the novel mutant NE protein with damaging functions and potential generation of the pre-leukemic HSPC clones with proliferative advantage and possible leukemic transformation. Adeno-associated virus (AAV)-based vector may be used for the delivery of the donor repair template and is considered safer than retroviral constructs. Two groups recently published successful gene deletions as a gene therapy approach to cure sickle cell disease, a common inherited blood disorder.^{17,18} It should be noted that AAV-based expression constructs may induce anti-viral host immune responses and may non-specifically integrate into the host genome.

In summary, we report here for the first time a method of CRISPR/Cas9 mediated *ELANE* gene deletion in hematopoietic stem cells and iPSC from CN patients harboring *ELANE* mutations. The *ELANE* gene deletion resulted in the increase of granulocytic differentiation to functional normal mature neutrophils in these patients *in vitro*. Therefore, CRISPR/Cas9 based gene knockout of *ELANE* in CN patients harboring *ELANE* mutation might be a useful treatment option especially in patients requiring high G-CSF dosages or do not responding to G-CSF at all. In addition, it remains to be investigated in subsequent clinical studies whether, in CN patients harboring *ELANE* mutations, the *ELANE* gene knockout would also abrogate the leukemogenesis.

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